

# Residues forming a hydrophobic pocket in ARF3 are determinants of GDP dissociation and effector interactions

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**Abstract** Three residues of human ADP-ribosylation factor 3 (ARF3) (F51, W66 and Y81) cluster into a hydrophobic pocket in the inactive, GDP-bound protein. Disruption of the hydrophobic pocket with mutations at these residues increased the rate of GDP dissociation and association, but not always that of GTP $\gamma$ S. Several of the same mutants were found to be defective, often selectively, in binding different ARF effectors in two-hybrid assays. These results highlight three features of these hydrophobic residues in regulating (1) the rate of GDP dissociation, (2) the conformational changes that promote GTP binding and (3) their role in binding target proteins. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** ADP-ribosylation factor; Regulatory GTPase; Effector interaction; Guanine nucleotide binding

## 1. Introduction

ADP-ribosylation factors (ARFs) are a family of 20 kDa GTP-binding proteins that regulate aspects of membrane traffic, including recruitment of protein coats [1–3], maintenance of Golgi integrity [4,5] and changing local lipid composition [6–10]. ARF cycles between a cytosolic, GDP-bound form and a membrane-associated, GTP-bound form. The functions of ARF are intimately associated with this cycle.

Two families of accessory proteins, the guanine nucleotide exchange factors (GEFs) and the GTPase-activating proteins control this cycle in cells. The exchange of bound GDP to GTP on ARF is catalyzed by the GEFs. ARF GEFs share a common domain of about 200 amino acids, which is homologous to the yeast Sec7 protein, referred as the Sec7 domain. The Sec7 domain interacts extensively with switch I and II of ARF and induces a conformation at switch I and II very similar to that seen in the GTP-bound structure but in the absence of any nucleotide [11]. The conformations of the switch I and II are very sensitive to the nucleotide binding state of ARF as both areas undergo a dramatic conformational change during the GDP/GTP cycle [11]. Switch I and II

are also binding sites for effectors [12]. We recently made the observation that mutations in two residues, F51Y and Y81C, cause ARF to bind GTP $\gamma$ S to higher stoichiometry [13]. When mapping F51 and Y81 on the GDP-bound ARF structure, we found that these two residues, together with W66, form a hydrophobic pocket between switch I and II. In the GTP-bound structure, these three residues are exposed on the protein surface and contribute to the formation of a hydrophobic patch that is shielded from the solvent by GEFs [11] and possibly by effectors, though comparable structures have been described. These observations led us to hypothesize that disruption of the hydrophobic pocket would facilitate the release of GDP and that binding of protein effectors likely involves direct, hydrophobic interactions with these same residues at the protein's surface to shield the hydrophobic side chains from solvent.

## 2. Materials and methods

### 2.1. Site-directed mutagenesis

The Quick Change site-directed mutagenesis kit (Stratagene) was used to introduce second site mutations in [Q71L]ARF3 at positions F51, W66 and Y81, in the vector pBG4D [14]. This allowed direct tests in two-hybrid assays. The complementary pairs of primers (31–32 bp) contained the desired mutation in the middle. The sequence of each mutation was verified by automated DNA sequencing.

### 2.2. Protein expression and purification

Mutant cDNAs were sub-cloned into the vector pET3C-[Q71L]ARF3 to allow bacterial expression, by switching each of the *NdeI/XmaI* fragments containing the mutations with the *NdeI/XmaI* fragment of [Q71L]ARF3. The resulting plasmids were transformed into BL21(DE3) cells. Protein expression and purification were performed as previously described [15] and yielded protein preparations that were >95% homogeneous.

### 2.3. GDP and GTP $\gamma$ S binding assays

The binding of [<sup>3</sup>H]GDP or [<sup>35</sup>S]GTP $\gamma$ S to ARFs was determined using the nitrocellulose filter trapping method to separate bound and free ligand, as described in Kahn et al. [16]. Each ARF (1  $\mu$ M) was incubated at 30°C with 10  $\mu$ M [<sup>3</sup>H]GDP or [<sup>35</sup>S]GTP $\gamma$ S (specific activity  $\approx$  30 000 cpm/pmol), in binding buffer (20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM MgCl<sub>2</sub>, and 100  $\mu$ g/ml bovine serum albumin) with 3 mM sonicated L- $\alpha$ -dimyristoyl phosphatidylcholine and 0.1% sodium cholate.

### 2.4. GDP dissociation

The rate of nucleotide dissociation was determined according to a previously described procedure [17]. Briefly, radiolabeled GDP was pre-loaded on ARFs by incubation of 1  $\mu$ M of ARF with 10  $\mu$ M [<sup>3</sup>H]GDP for 2 h at 30°C. Excess unlabelled GDP (1 mM) was then added at  $t=0$ , and duplicate samples were taken at the indicated times. The remaining bound GDP was determined by nitrocellulose trapping as described [16].

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### 2.5. Yeast two-hybrid assays

The interaction of ARF proteins with different binding partners were assayed using the yeast two-hybrid assay, as described previously [13]. Each ARF was expressed in yeast as a C-terminal fusion with the binding domain of GAL4 and each effector as an N-terminal fusion with the activation domain of GAL4. Colonies were replicated to nitrocellulose filters for assay using X-gal as a substrate and incubated for 30 min at 30°C.

## 3. Results

### 3.1. Disruption of the hydrophobic pocket by mutations at F51, W66 and Y81 promote GDP dissociation

A series of mutations were made at each of three residues (F51, W66 and Y81) and designed to represent variations in hydrophobicity, size and charge of the side chains. Mutant proteins were expressed in bacteria and purified to  $\approx 95\%$  homogeneity. Structures determined for ARF1 indicate that these three residues are organized into a hydrophobic pocket in the GDP-bound state and are all exposed on the protein surface in the GTP-bound conformation. The introduction of charged side chains were the most dramatic disruptants of the hydrophobic pocket but might be predicted to stabilize the activated form by decreasing exposure of hydrophobic side chains to the polar solvent. However, three of these mutants (F51R, Y81D and W66R) were insoluble in bacteria or so unstable as to prevent reliable determinations of nucleotide binding. The rates of GDP dissociation of the other mutants were determined as described in Section 2. As seen in Fig. 1A, all five changes at F51 resulted in proteins with an increased rate of GDP dissociation over that of the control ([Q71L]ARF3,  $k_{\text{off}} = 0.025 \text{ min}^{-1} \pm 0.001$ ). The largest change was seen in the F51D mutant for which the off rate for GDP was  $> \text{six-fold faster}$  ( $k_{\text{off}} = 0.154 \text{ min}^{-1} \pm 0.003$ ). A very similar pattern was seen at W66 with all changes resulting in higher rates of GDP dissociation and with the aspartate substitution giving the fastest rate, in this case a 20-fold increase ( $0.495 \text{ min}^{-1} \pm 0.055$ ; see Fig. 1B). All three mutants of Y81 also exchanged GDP at increased rates, comparable to most of the mutations at F51 and W66. Thus, mutations at each of these three residues yielded proteins with elevated rates of GDP dissociation and there was a correlation with the charged substitution giving the fastest rates of GDP dissociation, while the conservative substitutions, such as W66Y and F51Y gave smaller increases. Effects of the mutations at Y81 were found to be small, relative to the other two positions so will not be discussed further.

### 3.2. The binding of GDP but not always that of GTP $\gamma$ S is increased in response to the increased rate of the GDP dissociation

The binding of guanine nucleotides to regulatory GTPases is generally assumed to be limited by the off-rate of GDP, though this has been rigorously shown in only a few cases (e.g. [18]). Because nucleotide-free ARF proteins are extremely labile, it is not possible to obtain true on-rate measurements for guanine nucleotides. However, with the increases in the GDP off-rates we expect to see at least qualitatively corresponding increases in the GDP on-rates. This prediction was consistently observed with the mutations at F51 (see Fig. 2A) and Y81 (not shown) though the magnitude of changes was typically smaller for the association than for the dissociation. For example, each of the mutations at

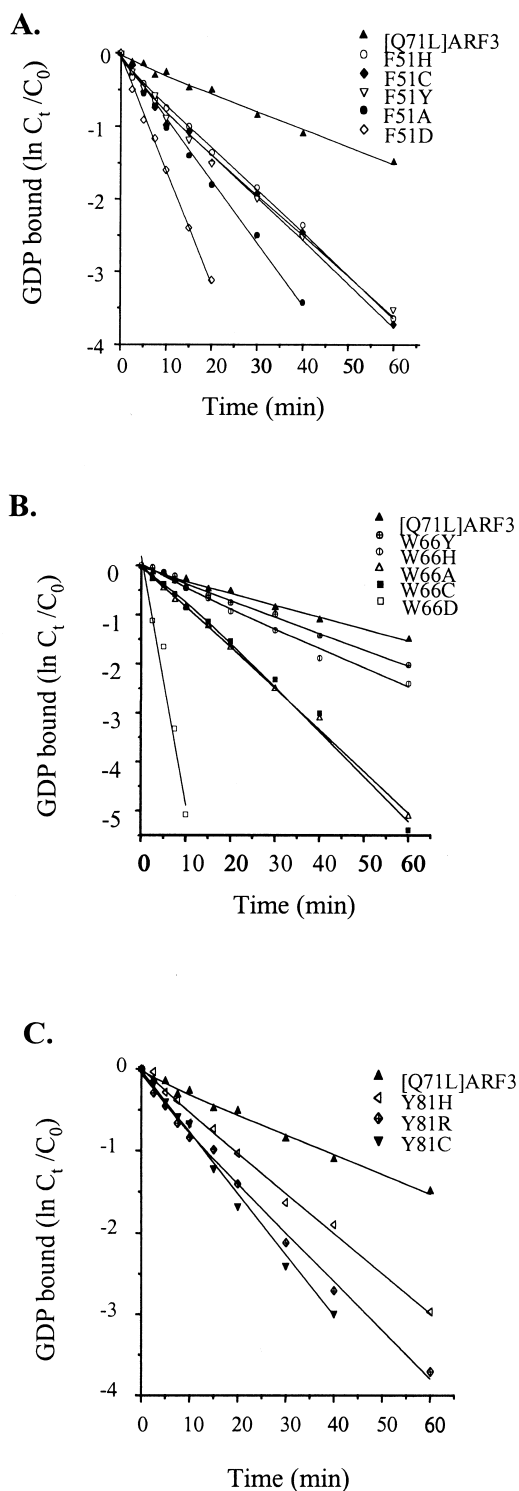


Fig. 1. Disruption of the hydrophobic pocket of human ARF3 by mutations at F51, W66 or Y81 promoted GDP dissociation. The GDP dissociation assay was performed as described in Section 2. The rate of dissociation,  $k_{\text{off}}$  ( $\text{min}^{-1}$ ) was determined by fitting the data to the equation  $\ln C_t/C_0 = -kt$ , where  $t$  is the time,  $C_t$  was the bound  $[^3\text{H}]\text{GDP}$  at indicated times,  $C_0$  was the bound  $[^3\text{H}]\text{GDP}$  at  $t=0$ . The dissociation curve of each mutant (as labeled in the figure) at F51 (A), W66 (B) and Y81 (C) are shown. The equilibrium binding of  $[^3\text{H}]\text{GDP}$  to each mutant was about 0.4–0.6 pmol GDP/pmol ARF. The data shown are averages of duplicate samples with a difference of less than 5%.

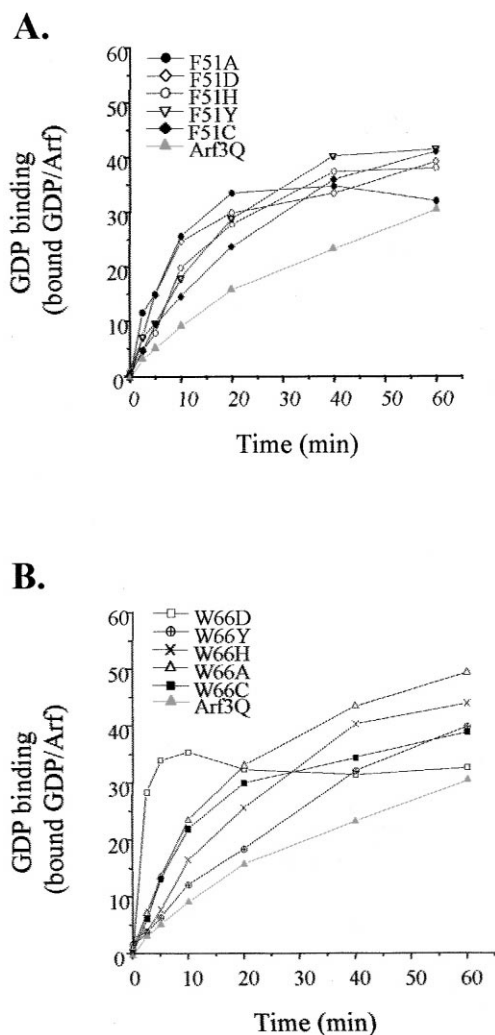


Fig. 2. Mutation of residues in the hydrophobic patch promote increased rates and extents of GDP binding. GDP binding was determined as described in Section 2. Note the qualitative similarities in results from Fig. 1 and this figure. Mutants at F51 (A) and W66 (B) are shown and are indicated in the figure.

residue 51 resulted in increased rates of binding of GDP with F51D and F51A giving the largest increases, about 2.5-fold. Similarly, the rank order is the same for GDP on- and off-rates among the mutations in W66 (see Fig. 2B), with W66D giving the fastest apparent on-rate but in this case only about seven-fold faster than the parental, [Q71L]ARF3, compared to the 20-fold increase in GDP dissociation. These results are consistent with the GDP-dissociation being rate limiting but they also suggest that association may be affected by other factors.

While GDP exchange is not known to require changes in protein conformation, the binding of GTP must be preceded by considerable rearrangement and movement of both side chains and secondary structures [11]. The similarities in the rate of binding of GDP and GTP $\gamma$ S among the mutations at F51 indicate that this side chain and those to which it was mutated were unlikely to be constrained during the conformational change that precedes GTP binding. However, some of the W66 mutants do not bind GTP $\gamma$ S more rapidly. On the contrary, the two mutants that exchange GDP the fastest, W66D and W66A, bind GTP $\gamma$ S quite poorly (see Fig. 3B).

We speculate that these changes at W66 both alter the hydrophobic pocket to decrease the stability of the GDP-bound ARF3 and interfere in some way with the conformational change required for GTP binding.

### 3.3. The same three residues are involved in selective binding to ARF effectors

The movement of the three hydrophobic residues and their exposure at the protein surface that accompanies GTP binding is central to the switching of this GTPase and its action as a regulator of cell functions. Their location near the two flexible switches makes them of likely importance to the binding of protein effectors. We tested this by using the yeast two-hybrid assay to determine the impact of mutations at F51, W66 and Y81 on the binding of five ARF effectors, POR1/Arfaptin [19,20], MKLP1 [14], LTA1 [21], GGA1 and GGA2 [22]. As seen in Table 1, different side chains at residue 51 yield proteins with differing abilities to bind effectors. For example, changing F51 to A, H or C disrupted the binding of all five effectors while the F51Y mutation preserved full binding to LTA1 and almost all binding of POR1. Mutations at W66 or Y81 also disrupted effector interactions but with distinct patterns of interactions. W66Y binds MKLP1 and LTA1 as well as [Q71L]ARF3 but does not interact with

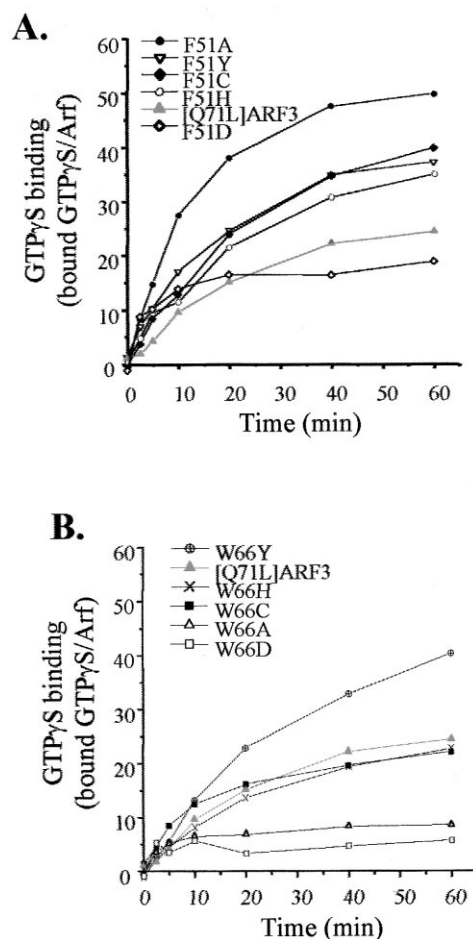


Fig. 3. [ $^{35}$ S]GTP $\gamma$ S binding to ARF3 mutants. [ $^{35}$ S]GTP $\gamma$ S binding was performed at 30°C, as described in Section 2. The binding curve of each mutant (as labeled in the figure) at (A) F51 or (B) W66 is shown. Samples were taken in duplicate with a difference of less than 5%.

the other three proteins. In contrast, Y81C binds each of the GGA proteins well but not the other three partners. Results described above indicate that all the mutants shown in Table 1 bind guanine nucleotides so are predicted to fold properly and were found expressed to similar levels in yeast. These results highlight the importance of these residues to effector binding.

#### 4. Discussion

Three residues of ARF3, F51, W66 and Y81, form a hydrophobic pocket in the GDP-bound protein and become exposed on the surface as a hydrophobic patch upon binding GTP. A series of mutations at each of these three residues were generated to disrupt the hydrophobic interactions. Their effects on GDP dissociation and subsequent GDP or GTP binding were tested. Disruption of the hydrophobic pocket by mutations at each residue resulted in increased rates of GDP dissociation in every case. However, while the binding of GDP was also increased this was not always the case with the activating nucleotides, e.g. GTP $\gamma$ S. Such mutations appeared to hinder the conformational changes required for GTP-binding. Mutations at each of these residues also interfered with the binding of ARF effectors, often in a specific fashion. We conclude that F51, W66 and Y81 play important roles in determining the rate of GDP dissociation, GTP binding and the binding of effectors. The coupling of ARF effector binding to changes in nucleotide affinities has been described recently [21] and may also involve one or more of these three hydrophobic residues. In contrast, because these residues are not exposed to solvent in the GDP-bound protein, they are predicted not to play a role in the binding of exchange factors.

The hydrophobic pocket is located a considerable distance from the nucleotide-binding site (see Fig. 4). How are conformational changes in the hydrophobic pocket propagated to the nucleotide-binding site? We propose that this is accomplished via effects on the position of strand  $\beta$ 2 and the  $\alpha$  helix (residue 30–37; switch I) whose N-terminus directly contacts the bound nucleotide. In the GDP-bound conformation, F51

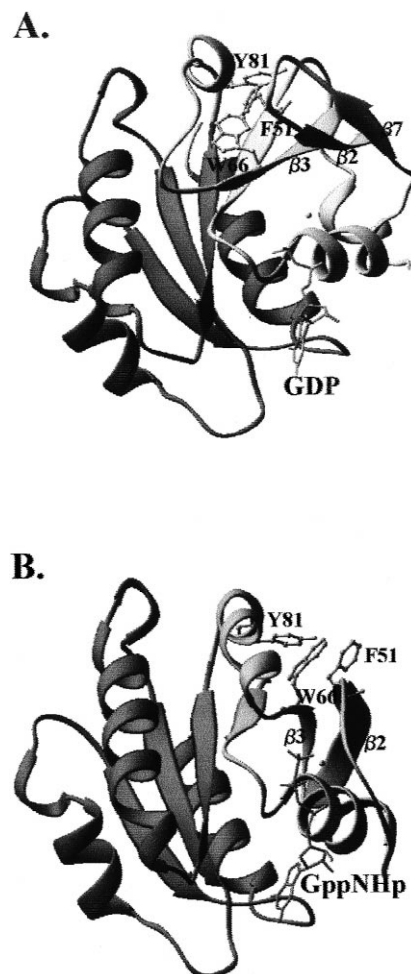


Fig. 4. Ribbon representations of GDP (A) and Gpp(NH)p (B) bound conformations of ARF1. The side chains of F51, W66 and Y81 are shown. Note the proximity of these three residues into a 'hydrophobic pocket' in the GDP structure and their surface location when GTP is bound. Note the distance to the bound nucleotides, also shown in the ball and stick model.

Table 1  
Interactions between ARF3 mutants and effectors in two-hybrid assays

Mutation	POR1	MKLP1	LTA1	GGA1	GGA2
[Q71L]ARF3	+++	+++	+++	+++	+++
[Q71L, F51A]ARF3	—	—	—	—	—
[Q71L, F51Y]ARF3	++	—	+++	—	—
[Q71L, F51H]ARF3	—	—	—	+	—
[Q71L, F51C]ARF3	—	—	—	—	—
[Q71L, W66Y]ARF3	—	+++	+++	—/+	—
[Q71L, W66H]ARF3	—	+	—	—	—
[Q71L, W66C]ARF3	—	++	—	—	—
[Q71L, Y81H]ARF3	—	+	++	+	—
[Q71L, Y81C]ARF3	—	—	—	+++	+++

Yeast strains harboring switch mutants (listed in the left column) were mated with strains harboring different partners (listed in the top row). The resulting diploid yeast strains, each harboring a set of mutant ARF3-BD and effector-AD plasmids, were assayed for  $\beta$ -galactosidase activity after lysis on nitrocellulose filters, as described in Section 2. '—' represents white color indicating the loss of interaction; '+' represents pale blue color indicating the retention of some binding; '++' represents more binding; '+++ represents full interactions, comparable to the [Q71]ARF3 positive control. Color development was detected by the eye, 30 min after the addition of the substrate, X-gal.

is located at the turn of strand  $\beta$ 2 and  $\beta$ 3; W66 is located in the middle of strand  $\beta$ 3 and Y81 is on the switch II helix (Fig. 4A). A unique feature of ARF-GDP, compared to other RAS-like GTPases, is the formation of the extra strand  $\beta$ 2 at switch I (Fig. 4A). Upon binding GTP, this extra strand becomes a loop and flips over aligning on the surface of the nucleotide (Fig. 4B). Disruption of the hydrophobic interactions among these residues may loosen the holding force of  $\beta$ 2, which in turn affects the position of the adjacent  $\alpha$  helix and weakens the binding of GDP. The larger effects of mutations at F51 and W66 than those at Y81 are predicted to be due to the lower hydrophobicity of the tyrosine and the relatively smaller distance moved by Y81 during GDP/GTP switching (Fig. 4A,B).

With increased rates of GDP dissociation, we would expect corresponding, at least qualitative, increases in the association of nucleotides. However, this was only seen for GDP binding to mutants at F51 and Y81. Although GTP $\gamma$ S binding is not increased as much as GDP dissociation on mutations at F51 and Y81, a similar rank order is still seen among the mutants. In contrast, the relationship between GDP and GTP $\gamma$ S association on mutations at W66 is more complicated. There is

reasonable agreement between effects of mutations on GDP dissociation and association but the effects on GTP $\gamma$ S binding were almost opposite to those on GDP binding/dissociation. This suggests that mutations at this residue have additional effects on GTP association such that the switch from the GDP- to GTP-bound forms is impaired.

The dramatic changes in conformation between the GDP- and GTP-bound states of ARF involve large movements of side chains and secondary structure [11]. Without the assistance of additional factors, particularly GEFs but also lipids and possibly protein effectors, ARFs would probably not be able to achieve the conformation to which GTP can bind with kinetics that would suffice for its role in cell regulation. Mutations that promote GDP dissociation but decrease GTP $\gamma$ S binding, such as W66D and W66A, may physically restrict the conformational changes required for activation. Alternatively, these mutations may destabilize the GTP-bound conformation. Activated ARF contains a hydrophobic patch that includes two pairs of hydrophobic interactions between strands  $\beta$ 2 and  $\beta$ 3, F51/V68 and V53/W66. Because the flexible loop of switch I is adjacent to  $\beta$ 2, we predict that these interactions stabilize switch I into an alignment close to the nucleotide. Disruption of the hydrophobic interactions by mutations at F51 or W66 may destabilize the switch I loop and thus impair formation of the GTP-bound conformation that is required for binding the activating nucleotide. The greater effects of mutations at W66 over those at F51 may result from their positions in the protein. W66 is located in the middle of a  $\beta$ -strand while F51 is at the turn between strand  $\beta$ 2 and  $\beta$ 3.

In summary, these three residues are all critical determinants of the rate of GDP dissociation but one, W66, is also important to the binding of GTP. Upon assuming the active conformation the same residues that are involved in the binding of GDP become exposed and help determine specificity in binding protein effectors.

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